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Distinct mechanisms mediate naïve and memory CD8 T-cell tolerance

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Peripheral tolerance to developmentally regulated antigens is necessary to sustain tissue homeostasis. We have now devised an inducible and reversible system that allows interrogation of T-cell tolerance induction in endogenous naïve and memory CD8 T cells. Our data show that peripheral CD8 T-cell tolerance can be preserved through two distinct mechanisms, antigen addiction leading to anergy for naïve T cells and ignorance for memory T cells. Induction of antigen in dendritic cells resulted in substantial expansion and maintenance of endogenous antigen-specific CD8 T cells. The self-reactive cells initially exhibited effector activity but eventually became unresponsive. Upon antigen removal, the antigen-specific population waned, resulting in development of a self-specific memory subset that recalled to subsequent challenge. In striking contrast to naïve CD8 T cells, preexisting antigen-specific memory CD8 T cells failed to expand after antigen induction and essentially ignored the antigen despite widespread expression by dendritic cells. The inclusion of inflammatory signals partially overcame memory CD8 T-cell ignorance of self-antigen. Thus, peripheral CD8 T-cell tolerance for naïve CD8 T cells depended on the continuous presence of antigen, whereas memory CD8 T cells were prohibited from autoreactivity in the absence of inflammation.

autoimmunity | AIRE | transgenic mouse

Immune tolerance is mediated by multiple mechanisms. Central tolerance that results in deletion of high-avidity autoreactive T-cells occurs in the thymus during T-cell development (1, 2). The deletion process is rendered more robust by the action of the Aire system, which promotes expression of nonthymic, tissue-specific proteins in thymic medullary epithelial cells (3). However, this process is not infallible and self-reactive clones escape to the periphery where other control mechanisms come into play. Peripheral tolerance can be mediated through regulatory T cells (Treg) (4), deletion (5), or the induction of an unresponsive state known as anergy (5, 6). Aire also operates in the periphery but to what extent it contributes to each of these tolerance mechanisms is not yet known (7, 8). In addition, T cells specific for self-antigens that are developmentally regulated and whose expression is not controlled by Aire may also be present in the periphery (1). Many studies using a variety of systems have focused on the induction of tolerance of adoptively transferred naïve monoclonal CD4 and CD8 T cells. Few reports have analyzed tolerance induction of endogenous T cells, and fewer still have analyzed the potential for the development of tolerance in endogenous memory T cells (9, 10). Memory T cells may be more relevant to the development of some autoimmune diseases because preexisting cross-reactive memory cells may react with tissue-specific antigens through molecular mimicry (11, 12). However, little is known regarding the mechanisms by which memory T cells may be tolerized. For example, after the transfer of memory CD8 T cells to antigen-bearing hosts, the mice become unresponsive, but whether this unresponsiveness is due to anergy or deletion could not be determined (9), whereas injection of soluble antigen deletes endogenous memory cells (13).

Transgenic systems in which model antigens are expressed under systemic (14, 15) or tissue-specific promoters (16, 17) have been

used to study T-cell tolerance. Transgenic mice have also been developed whereby antigen is expressed exclusively by dendritic cells (DCs) (18) that, in some cases, can be temporally controlled (19). These models rely heavily on the analysis of adoptively transferred naïve or memory monoclonal TCR transgenic T cells because the endogenous antigen-specific T-cell repertoire is dramatically altered through deletion via central tolerance. In a more elegant system, administration of tamoxifen results in antigen expression in DCs, but this expression is irreversible (10). Expression of antigen in resting DCs using this model results in tolerance induction without apparent expansion of antigen-specific CD8 T cells. Again, whether anergy or deletion of the endogenous CD8 T cells is the mechanism of tolerance is not known. Even in a case where antigen induction is reversible, adoptive transfer of TCR transgenic T cells was used, likely due to leaky expression of the transgene, perhaps through the action of Aire (20). We now report an inducible and reversible system in which endogenous CD8 T cells are ignorant of transgenic antigen. The system allowed us to observe endogenous CD8 T cells responding to self-antigen and, importantly, allowed the generation of endogenous memory CD8 T cells before the induction of autoantigen. The results reveal surprising characteristics of the endogenous naïve and memory CD8 T-cell responses to developmentally regulated antigen.

Results

Development of an Inducible Antigen System with High Fidelity. We sought to devise a transgenic system that would allow inducible antigen expression while maintaining immune system ignorance. Three transgenic elements were used to control antigen expression (Fig. S1). The model antigen was a fusion protein (SED) that encoded an ovalbumin-derived peptide 257–264 [SIINFEKL (OVA₂₅₇)], an I-E alpha peptide (aa 52–68) and DsRedII. The gene encoding this protein was linked to the minimum CMV promoter under control of the tetracycline operator (tetOSED) (21). The reverse tetracycline transactivator (rtTA) (22) was expressed under control of the CD11c promoter (23), restricting antigen expression to DCs. Tight control of antigen expression was achieved through inclusion of a tetracycline-controlled transcriptional suppressor (tTS) that was expressed under control of the β -actin promoter (24).

To test fidelity of the system, CFSE-labeled TCR transgenic OT-I T cells specific for SIINFEKL (25) were used to assay antigen

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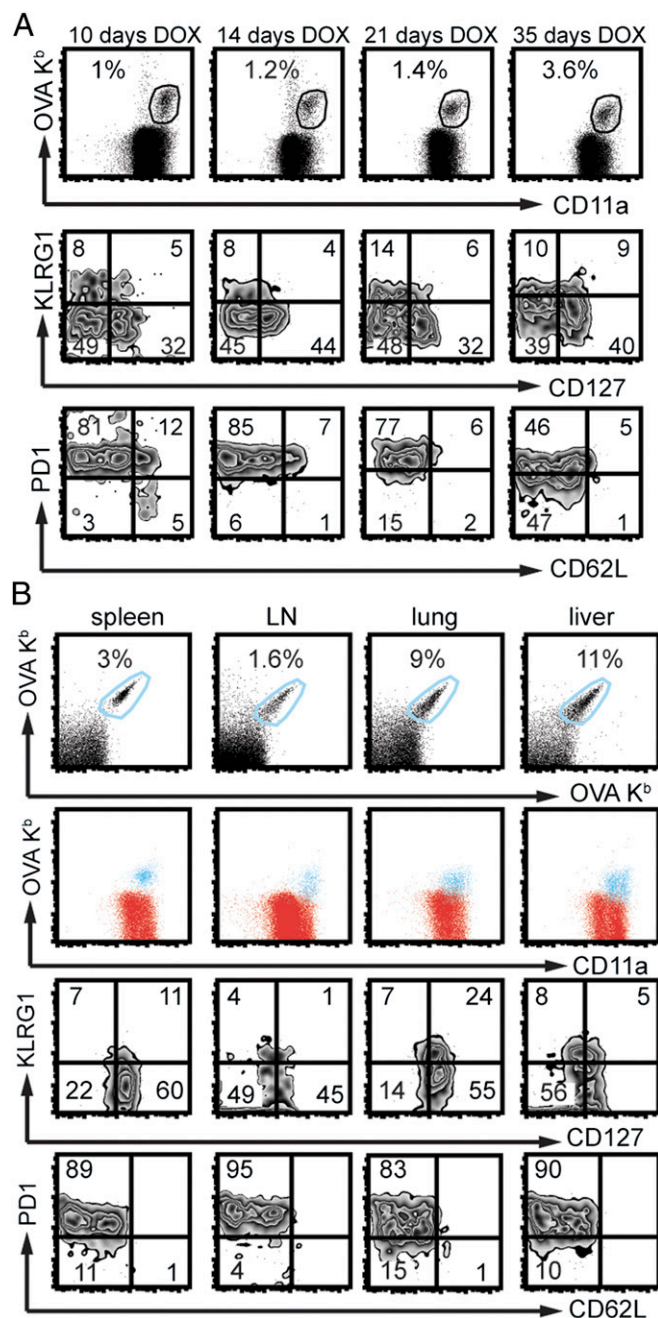


Fig. 3. Analysis of self-specific CD8 T cells in lymphoid and nonlymphoid tissues. (A) Lymphocytes from secondary lymphoid organs were stained as in Fig. 2A, but without magnetic sorting. (B) Lymphocytes from CST BMC mice were isolated from indicated organs after 35 d of doxycycline treatment, stained as indicated, and analyzed by flow cytometry. These experiments were performed at least two times with a minimum of three mice per group.

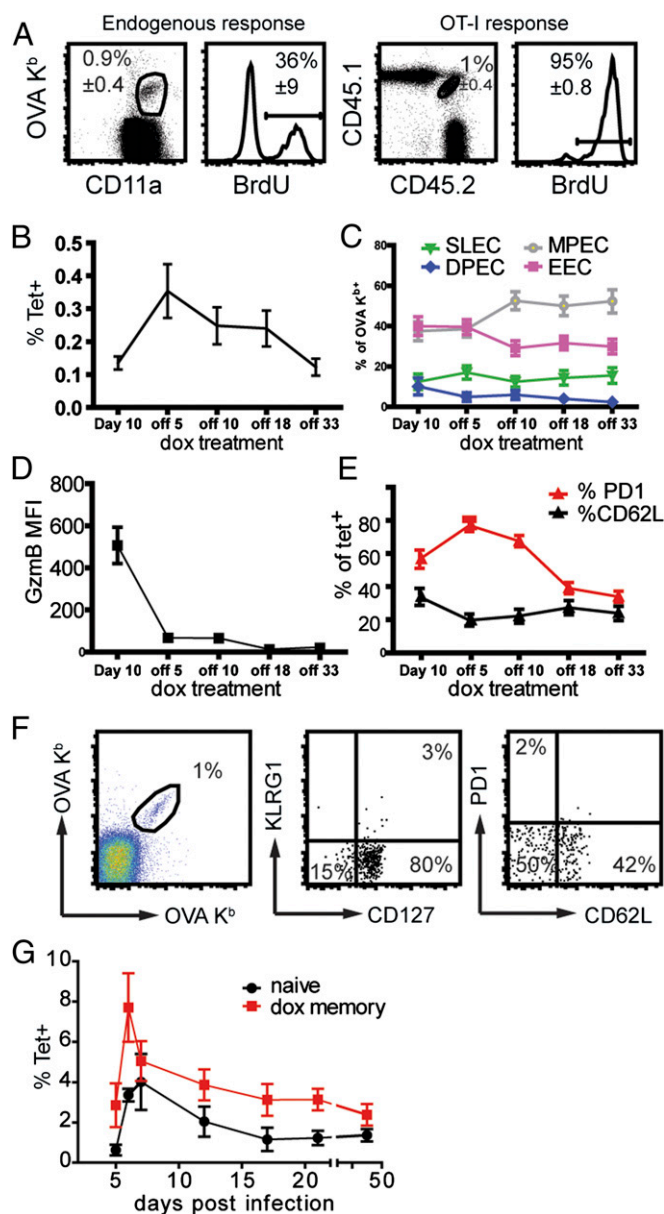
10 had a subset of responding cells in the blood down-regulated CD127, and this proportion remained at ~50% throughout, whereas few cells up-regulated KLRG1 (Fig. 3A). Similar results were obtained when cells from lymphoid and nonlymphoid tissues were analyzed 35 d after antigen induction (Fig. 3A and B). From day 10 on, most cells expressed high levels of the negative regulator PD-1 and had down-regulated CD62L (Fig. 3A and B). Nonetheless, 10 d after antigen induction the cells expressed granzyme B and killed target cells in vivo (Fig. S3B and C). By

day 35 after induction, little cytokine production (IFN γ , IL-2, TNF) was evident from peptide-stimulated cells (Fig. S3A). Thus, antigen induction on resting DCs resulted in robust CD8 T-cell expansion and acquisition of effector function. Nevertheless, the eventual outcome of continuous antigen encounter was the development of anergy and maintenance of cell numbers.

Maintenance of Self-Specific CD8 T Cells Requires Antigen. We next tested whether the long-lived self-specific CD8 T cells were proliferating in response to antigen. To this end, CST BMC were administered doxycycline for 45 d with BrdU given during the last week and then splenic tetramer⁺ cells were analyzed. BrdU was incorporated by ~30–40% of the antigen-specific CD8 T cells (Fig. 4A). Nearly all naive OT-I cells transferred to the same mice incorporated BrdU (Fig. 4A). Thus, the maintenance of cell numbers appeared to be due to attrition along with continued proliferation.

We also queried whether antigen was required for the maintenance of the responding CD8 T cells. Mice were doxycycline-treated for 10 d followed by doxycycline removal for 30 d. At 5 d after doxycycline withdrawal, the frequency of OVA-specific cells in the blood increased ~twofold (Fig. 4B), which may be due to release of T cells from the lymphoid tissues as a result of disruption of T-cell–DC interactions. From this timepoint on, the frequency of OVA-specific CD8 T cells slowly waned and the cells regained CD127 expression but rapidly lost GrzB expression with gradual loss of PD-1 expression (Fig. 4B–E). By 33 d after antigen removal, 50–60% of the tetramer⁺ cells were memory phenotype (CD127⁺KLRG1[−]) with a smaller population (~30%) lacking both CD127 and KLRG1 (Fig. 4C). Tetramer enrichment at 35 d after doxycycline cessation revealed that ~1,000 memory phenotype T cells remained in the sampled secondary lymphoid organs (Fig. 4F). These cells were CD127⁺KLRG1[−] and heterogeneous for CD62L expression (Fig. 4E), suggesting that both central and effector memory cells had been produced. We therefore tested whether such memory cells could respond to VSV-SED infection. In mice previously doxycycline treated, the OVA-specific response peaked earlier, was of a higher magnitude, and generated more secondary memory cells than the response in naive mice (Fig. 4G). Interestingly, OVA-specific self-antigen-induced memory cells displayed a higher avidity for H-2K^b/OVA₂₅₇ compared with uninduced mice ($T_{1/2}$ = 18 min compared with 7 min) (Fig. S4), similar to memory cells responding to infection (26, 33). Thus, even in response to self-antigen, “avidity maturation” (26) occurred. These results indicated that antigen addition maintained anergy, whereas removal of antigen resulted in development of memory cells.

Preexisting Antigen-Specific Memory CD8 T Cells Respond Poorly to Self-Antigen. The ability to generate normal pathogen-specific responses before antigen induction in our system afforded us the ability to examine memory T-cell responses to self-antigen. CST BMC mice were infected with VSV-SED, and memory CD8 T cells were allowed to develop. Induction of antigen in naive mice resulted in expansion of antigen-specific cells and incorporation of BrdU by most cells (Fig. 5A). In the case of memory cells, in the absence of doxycycline, ~10% of the memory CD8 T cells incorporated BrdU over 7 d, indicative of normal homeostatic proliferation (Fig. 5B). Surprisingly, when antigen was induced, antigen-specific memory CD8 T cells did not increase in number (Fig. S5) and only ~30% of the cells incorporated BrdU (Fig. 5B). No phenotypic changes indicative of antigen recognition (CD69 or PD-1 up-regulation or TCR down-regulation) were detected. Even when doxycycline was administered for >30 d, no further increase in memory cell proliferation was noted. The memory cells readily reactivated in response to OVA-expressing vaccinia virus infection, which induced robust BrdU incorporation (Fig. 5C) and an increase in cell numbers (Fig. S5). We then tested whether the addition of inflammatory signals



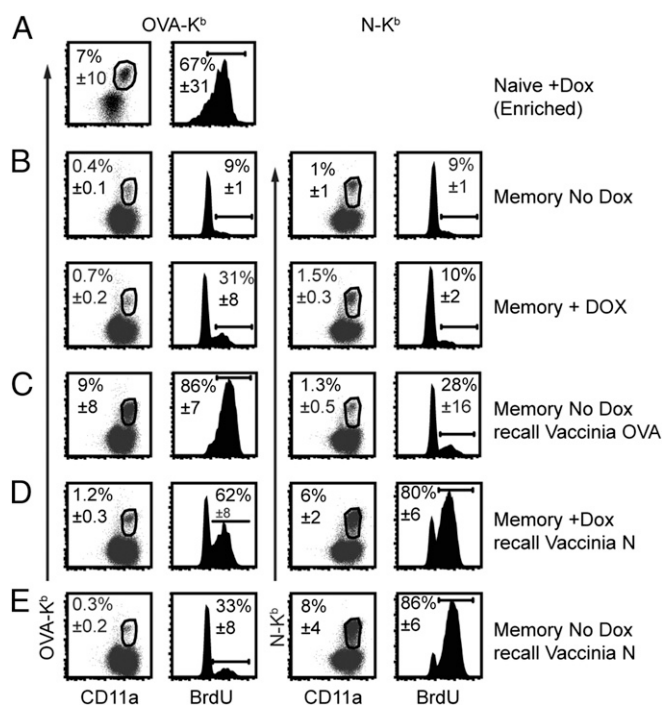
could overcome the relative ignorance of memory CD8 T cells for self-antigen. When doxycycline was administered along with infection by vaccinia virus lacking OVA, BrdU incorporation increased ~twofold (from ~30–60%) compared with control

mice without doxycycline treatment (Fig. 5D). This increase in proliferation did not, however, result in an increase in cell numbers (Fig. S5). In addition, in the absence of doxycycline, infection with vaccinia virus without OVA resulted in an increase in memory CD8 T-cell proliferation from ~10% to ~30% (compare Fig. 5B–E), likely due to bystander proliferation (34). Thus, inflammation promoted antigen recognition by memory cells to a limited extent, but far from the level to which naïve CD8 T cells responded to self-antigen.

Discussion

One advantage of the inducible antigen model described here is the ability to add or subtract antigen at will. Although we and others have described systems where self-antigen is constitutively expressed or can be irreversibly induced, in most cases, they suffer from leaky promoter-mediated antigen expression or AIRE-mediated expression resulting in central tolerance. The inclusion of the transcriptional silencer element in our system blocked aberrant antigen expression, thereby allowing de novo antigen induction and withdrawal of antigen. Thus, this system allowed examination of the endogenous naïve and memory CD8 T-cell response to temporally regulated self-antigen, without the need for adoptive transfer of TCR transgenic T cells.

Our findings identified two distinct mechanisms for endogenous naïve and memory CD8 T cells to maintain self-tolerance. Naïve CD8 T cells responded robustly to self-antigen via direct presentation by DCs, resulting in transient effector activity



followed by anergy induction in the apparent absence of deletion. The expansion observed for naïve cells within 10 d after antigen induction was likely primarily due to the recruitment of mature naïve CD8 T cells into the response. Nevertheless, over the course of self-antigen induction, some newly minted T cells from the thymus may also have been recruited into the response, which could explain the increased numbers of antigen-specific CD8 T cells observed after 35 d of antigen induction. This scenario would presumably represent the normal course of events for tolerance induction to developmentally regulated and/or tissue-specific antigens in an intact host. However, it is possible that at later times after antigen induction, deletion was occurring simultaneously with new input from recent thymic emigrants. It might also be expected that deletion of antigen-specific cells in the thymus would occur in response to DC-expressed antigen, but further studies will be needed to dissect this issue. Interestingly, the responding T cells were initially induced to become functionally active, as evidenced by their ability to mediate antigen-specific cytotoxicity (Fig. S3B). However, DC numbers remained stable in antigen-induced mice and, indeed, antigen continued to be presented because transferred OT-I cells were able to respond in the context of an ongoing endogenous response (Fig. 2C). The eventual induction of anergy also correlated with the sustained expression of PD-1 (Fig. 3A), an inhibitory receptor associated with T-cell exhaustion and chronic viral infection (35), but other exhaustion markers were not induced. Thus, signaling via PD-1 may directly inhibit the effector function of the induced T cells.

Of particular interest was the finding that the immune response to short-term self-antigen exposure resulted in the generation of memory CD8 T cells. In fact, self-antigen encounter selected high-avidity memory cells that were able to mount a secondary response to subsequent challenge. These data potentially hold implications for the induction of autoimmunity because a second exposure to antigen in the face of an inflammatory event could result in tissue damage. This finding also contrasts with our previous observations involving central tolerance, where high-avidity CD8 T-cell clones undergo deletion (26). Similar to virus-induced memory, the self-antigen-induced memory cells up-regulated IL-7R and lost PD-1 expression, but unlike a pathogen-induced response, formation of KLRG1⁺ short-lived effector cells was limited, no doubt due to the absence of inflammation (36, 37). The addition of inflammatory signals during self-antigen induction may thus change the shape of the observed response as we have observed in an adoptive transfer system (38).

Remarkably, although naïve CD8 T cells underwent vigorous expansion in response to self-antigen, preexisting endogenous memory CD8 T cells largely ignored the antigen, although inflammation partially overcame this ignorance. This result appeared to fly in the face of the long-held tenet that memory T cells are more readily triggered compared with naïve T cells. Recent results in virus infection systems also suggest that memory CD8 T cells

may have a preference for particular APC types (39, 40). Our model predicts that memory T cells have additional requirements for activation when presented self-peptides either by sequestration of memory T cells from DCs or by a need for specific costimulation that is not provided by self-antigen in the absence of inflammation. The mechanism we describe may provide a means by which memory T cells that cross-react with self-antigens can be maintained for their protective benefits without the danger of autoimmunity.

Materials and Methods

Mice. Transgenic mice were generated separately by microinjection of fertilized embryos with constructs containing CD11c-rtTA (C) and tetO-SED (S) (26). Triple transgenic CST mice were generated by breeding double transgenic CS mice with mice expressing tTS (T) under control of the β -actin promoter (24). All animal protocols were approved by the Animal Care Committee at University of Connecticut Health Center.

Bone Marrow Chimera Production and Analysis. CST chimeras were produced by transferring $\sim 1 \times 10^6$ anti-Thy1 and complement treated BM cells from CST mice into lethally irradiated host mice. Some BMC were generated by mixing CST bone marrow and wild-type bone marrow cells at indicated ratios. For antigen induction, doxycycline (Sigma) was administered in drinking water (10 μ g/mL) with 5% (wt/vol) sugar (Domino). To validate the fidelity of antigen induction, 5×10^5 CFSE-labeled (Invitrogen) naïve Rag^{-/-} CD45.1.2 OT-I cells were injected i.v. and analyzed by flow cytometry 6 d later. To test for proliferation of CD8 T cells, BrdU (Sigma) was administered at 0.8 mg/mL in drinking water with or without doxycycline. Incorporation of BrdU was determined by using the BD Pharmingen flow kit. To isolate small numbers of antigen-specific CD8 T cells, magnetic tetramer enrichment of T cells was performed as described (29).

Flow Cytometric Analysis. Staining was performed by resuspending cells at 10^6 to 10^7 cells per mL in PBS containing 0.2% BSA and 0.01% NaN₃. OVA and N-specific T cells were identified by staining with APC-labeled H-2K^b tetramers containing the SIINFEKL epitope (OVA-tetramer) or VSV-nucleoprotein peptide (N-tetramer) for 1 h at room temperature in the presence of anti-CD8 (clone 53.6.7), followed by staining with antibodies specific for the indicated molecules at 4 °C for an additional 30 min (all antibodies were purchased from Biolegend or eBioscience). Cells were washed and fixed with 3% paraformaldehyde/PBS, fluorescence intensities were measured with a LSR II (BD Biosciences), and data were analyzed by using FlowJo software (Tree Star). Avidity of T-cell populations was evaluated by decay of tetramer binding, as described (26).

Analysis of Memory CD8 T Cells. To generate memory cells, CST BMC mice were infected i.v. with 1×10^5 pfu VSV-SED (41). Sixty days later, the mice were given doxycycline and subsequently analyzed as detailed in the text.

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